Grant Proposal

Project Number: T-103-R-1

Project Title: Survey and Risk Assessment of the Salamander-killing Chytrid Fungus in Illinois

Background information

Batrachochytrium salamandrivorans* (*Bsal*) has been described in 2013** (Martel et al. 2013) and is **associated with salamander die-offs** in the Netherlands and Belgium (Spitzen-van der Sluijs et al. 2013). The current die-offs concern a single species, but experimental trials show that many European and **North American salamanders are highly susceptible to infection by *Bsal (Martel et al. 2014). This finding is highly relevant because the United States and Mexico host a large proportion of global salamander biodiversity. **Should *Bsal* spread in North America, it could cause the collapse of native communities of salamanders**.

Bsal grows best at moderate temperature, similarly to *Bd*, but the optimum seems to be lower for *Bsal*. For example, growth of *Bsal* on infected European fire salamanders is faster and causes greater host mortality at 15˚C than at 20˚C (Blooi et al. 2015). Such low growth temperatures do not bode well for salamander-rich communities, many of which occur along cold streams and moist riparian habitats of temperate forests in the US. On the other hand, *Bsal* does not grow at 25˚C, and salamanders clear infection after being kept at 25˚C for 10 days (Blooi et al. 2015). Assuming such temperature is within the range tolerated by hosts, heat treatment could therefore be an effective strategy to clear infection in wild salamanders rescued during a *Bsal* outbreak.

Global surveillance of *Bsal* is a conservation priority. We have missed the earliest epizootics of *Bd* and ignore the origin of the strains that caused species extirpations, but we might still have a chance to locate the source of the epizootic strain of *Bsal* and to contain its spread to other regions. The **Chinese fire belly newt (*Cynops orientalis*) is an ancient and immunocompetent host for *Bsal*** (Martel et al. 2014) and could be a carrier of *Bsal*. Worryingly, **fire belly newts are among the most commonly imported salamander in the US** (Kolby et al. 2014). Therefore there is **great risk of pathogen spillover** from the live amphibian trade, as well as from pet owners who release infected newts outside of their native ranges.

Bd has set a painful example of how disease can cause irreversible biodiversity loss. **The live amphibian trade is a likely vector of *Bd*, *Bsal*, Ranavirus and other diseases and parasites that endanger immunologically naïve species**. Millions of wild caught amphibians are traded every year across countries and within the US as pets, food, fishing bait, or specimens for education and research. There is an **urgent need to regulate such live trade and to screen live amphibians for infection with diseases known to cause population declines and extirpations**. We have the **capacity to develop cost-effective surveillance programs that can quickly detect infected individuals**. Stringent legislation, globally coordinated efforts and collaborations between government agencies, conservation organizations and researchers will reduce the probability of pathogens being introduced into new areas.

Need: We need to **know whether a recently discovered and highly virulent pathogenic fungus is found in populations of salamanders in IL**. The fungus was discovered in 2013 and has been named *Batrachochytrium salamandrivorans* (*Bsal*) because it causes extensive skin destruction and rapid mortality in infected salamanders. **This salamander-killing fungus has extirpated populations of salamanders in northwestern Europe** (Martel et al. 2013, Spitzen-van der Sluijs et al. 2013, Martel et al. 2014). We need to **assess whether virulence is similarly high for native Illinois salamanders**. The related chytrid fungus *B. dendrobatidis* (*Bd*) has caused mass die-offs and the extinction of hundreds of species of amphibians worldwide (Kilpatrick et al. 2010, Fisher et al. 2012), while Ranavirus outbreaks

are one of the most common causes of mass die-offs in amphibian populations in North America (Fey et al. 2015).

All 19 species of salamanders (including the eight salamander species in Greatest Need of Conservation; Appendix 1) in IL could be affected by the introduction of this pathogen into the state. Disease outbreaks could be especially devastating for populations of species with restricted geographic distribution, such as the Silvery and Jefferson salamanders, and the Spotted Dusky salamander. More generally, the spread of this virulent disease in North America, the region with the richest diversity of salamanders, would have dramatic consequences on global amphibian biodiversity and is a pressing conservation priority (Martel et al. 2014).

Purpose and objectives: The main objectives of the proposed study are to **determine whether a highly virulent salamander pathogen is present in Illinois, and to quantify its potential threat for native species in Greatest Need of Conservation**. This project will directly contribute to action items in the Illinois Wildlife Conservation Plan that cover Wetlands Campaign, Invasive Species, Monitoring and Research of Wildlife Disease, and Monitoring and Research on Amphibian Species of Conservation Priority (http://dnr.state.il.us/ORC/WildlifeResources/theplan/final/). As outlined in the IWCP (pg. 31), "Fourteen of Illinois' 41 amphibian (34%)" species are considered "Species in Greatest Need of Conservation, eight are threatened or endangered, and 1 has a Global Conservation Rank of G3."

The **specific goals** are to:
1. Quantify **prevalence and intensity of infection by *B. salamandrivorans* (*Bsal*) in native populations** of salamanders in Illinois
2. Quantify prevalence and intensity of infection by *Bsal* in **captive populations of salamanders** in Illinois
3. Determine the **geographic distribution of *Bsal* in Illinois**
4. Identify **potential routes of pathogen introduction** and generate recommendations to avoid introduction and spread of disease
5. Assess the **susceptibility to infection** (i.e., reduction in survivorship) for salamander species of Greatest Need of Conservation
6. Quantify **prevalence and intensity of infection by *B. dendrobatidis* (*Bd*) and Ranavirus (Rv)**, two other pathogens associated with amphibian population declines and extinction, by using the same diagnostic that quantifies infection by B*. salamandrivorans*.

Results or benefits expected: This project will directly contribute to action items in the Illinois Wildlife Conservation Plan that cover Wetlands Campaign, Invasive Species, Monitoring and Research of Wildlife Disease, and Monitoring and Research on Amphibian Species of Conservation Priority (http://dnr.state.il.us/ORC/WildlifeResources/theplan/final/).

All salamander species in Illinois, and particularly those in Greatest Need of Conservation, will benefit from a survey and risk assessment for *B. salamandrivorans*. The scope of our surveys include **both wild populations of native species**, possibly not infected at the moment, and **captive populations of exotic salamanders that could introduce the pathogen in IL**. Moreover, **our novel protocol will allow simultaneous detection of two other amphibian pathogens associated with population declines**.

Specifically, our results will answer the following questions: (1) does *Bsal* occur in IL, and if so, in which species and localities? (2) how virulent is *Bsal* for the four *Ambystoma* species in Greatest Need of Conservation? (3) assuming *Bsal* has virulence similar to what observed in Europe, which levels of infection are associated with high mortality in *Ambystoma* species? (4) what are the prevalence, intensity of infection and distribution of *Bd* and Rv in salamanders in IL?

Experiment and survey results will be made available through technical reports, scientific articles, and a publicly accessible mapping database. The technical report will quantify probability of mortality following *Bsal* infection (odds ratio), visualize distribution and prevalence of co-infection by pathogens known to cause population declines, and generate recommendations for minimizing the risk of introduction and spread of *Bsal* in IL.

Approach: We have developed a **multiplex protocol to simultaneously test for the presence and quantify levels of infection of *Bsal* and *Bd* through real time Polymerase Chain Reaction**. Previous protocols have used standards from DNA extracted from live cultures (Blooi et al. 2013), but our **novel protocol uses strands of synthetic DNA that contain target gene sequences of the two chytrid pathogens (*Bsal* and *Bd*) and Ranavirus** (Figure 2). Time and costs per sample are significantly reduced because a single reaction can quantify infection levels for the three pathogens, and no live cultures need to be maintained to generate standards. We will amplify pathogen DNA recovered from swabs collected by gently rubbing the skin of salamanders.

Our surveys will target three groups: (1) salamander species in Greatest Need of Conservation; (2) populations in regions with highest salamander species richness in IL and (3) live trade of captive salamanders in pet stores and terrarium shows. We will collaborate with researchers already surveying populations to avoid duplication of survey efforts whenever possible.

Our infection trials will assess susceptibility to infection by *Bsal* in species in the four *Ambystoma* species of Greatest Need of Conservation, depending on availability of eggs. We will collect eggs and rear them to metamorphosis in the lab, which significantly reduces any potential effect on population abundance, and guarantees that none of the individuals used in experiments are exposed to *Bsal* or other pathogens during development. After rearing larvae, we will use recent metamorphs, which are known to be most susceptible to chytridiomycosis, and compare infection levels and survivorship between animals exposed to *Bsal* and control animals with no exposure. This experiment will assess the threat that *Bsal* could pose to these native salamanders.

Useful life: N/A

Geographic location: The project will focus on four regions supporting wild populations of the four species of *Ambystoma* in greatest need of conservation (Figure 1), and on pet stores and herpetological fairs offering live specimens of salamanders throughout the State of Illinois.

Field work locations (Figure 1)

Ambystoma laterale (Blue-spotted salamander) populations: at least three known sites of *A. laterale* will be surveyed in the Northeastern Morainal Natural Division (Mchenry, Lake, Cook, Dupage, Will). Additional target species are *A. maculatum*, *A. texanum*, *A. tigrinum*, *Eurycea cirrigera*, *Hemidactylium scutatum*, *Plethodon cinereus*, *Notophthalmus viridescens*.

A. platineum (Silvery salamander) populations: at least two and up to three known sites of *A. platineum* will be surveyed in Kickapoo State Park, Vermilion Co. and in Crawford Co. Additional target species are *A. maculatum*, *A. opacum*, *A. texanum*, *A. tigrinum*, *Eurycea cirrigera*, *Hemidactylium scutatum*, *Plethodon cinereus*, *P. glutinosus*, *Necturus maculosus*.

A. jeffersonianum (Jefferson salamander) populations: at least two and up to three known sites of *A. jeffersonianum* will be surveyed in Clark Co. and Edgar Co. Additional target species are *A. maculatum*, *A. texanum*, *A. tigrinum*, *Eurycea cirrigera*, *Plethodon cinereus*, *P. glutinosus*, *Necturus maculosus*.

A. talpoideum (Mole salamander) populations: at least three known sites of *A. talpoideum* will be surveyed in Jackson Co., Union Co., Alexander Co., Johnson Co., Massac Co. or Pope Co. Additional target species are *A. maculatum*, *A. opacum*, *A. texanum*, *A. tigrinum*, *Desmognathus conanti*, *Eurycea*

cirrigera, *E. longicauda*, *E. lucifuga*, *Plethodon dorsalis*, *Plethodon glutinosus*, *Necturus maculosus*, *Notophthalmus viridescens*, *Siren intermedia*.

Pet stores, pet owners and herpetological fairs

Pet owners will be contacted through herpetological societies (Chicago Herpetological Society, Central Illinois Herpetological Society), and invited to send swabs of their salamander pets to the lab. We will make swabbing kits and information on salamander-killing fungus available to interested pet owners. Pet stores will be contacted by phone, email or mail before visiting to make sure the owner agrees to testing of animals. We expect most of these pet stores to be located in major urban areas, especially Chicago. We will make swabbing kits and information on salamander-killing fungus available to interested pet store owners.

We will visit herpetological fairs (NARBC, Tinley Park; ReptileFest, Chicago) and request permission to swab any salamander that is available for sale. We will also distribute information (small brochure) on the salamander-killing fungus.

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Program income: N/A

Budget Narrative:

Salaries and Wages: The proposed activities are labor intensive. Therefore, the bulk of the requested funds will be used to support a graduate student in the Department of Zoology at SIUC for 23 months. Graduate stipends at SIUC are $9,628 in year 1, $19,836 in year 2, and $8,513 in year 3 assuming an anticipated increase of 3% per year. We will rely on undergraduate students to assist with laboratory work, as we have done in the past. A variable number of students will be paid student researchers hired through the Center for Undergraduate Research & Creative Activities (curca.siu.edu). The PI also regularly trains several volunteer undergraduate students every semester. The undergraduate(s) will assist primarily in fieldwork, lab work (molecular analyses, real-time PCR), and data entry. The PI will use his SIUC salary as a match and will thus receive no federal funds. SIUC will contribute 18 months per 0.0188 of year salary for Dr. Alessandro Catenazzi for a total of $2,411.

Fringe Benefits: A primary care fee is also requested for each year the graduate student will be employed as a requirement of their contract. Primary care fees at SIUC are $116 in year 1, $311 in year 2, and $124 in year 3 for a total $551 over the course of the project. The PI will also use his SIUC fringe benefits as match and will thus receive no federal funds. Fringe benefits for Dr. Alessandro Catenazzi total $1,319 for both retirement and medical.

Travel: We request a total of $4,576 federal dollars for travel expenses to pay for the gas and mileage, meals and lodging for two years of field work. Based on an estimate of 3 visits to each of the 3 regions (2-3 sites per region) per year to obtain the necessary number of samples, and the assumption that the southern sites are ~50 miles away (3*50*2 = 300 miles at 0.575/mi; requiring 3 days of per-diem

@$28/day = $84), costs for Southern sites will total $256.50/year; the Central-Eastern sites are ~285 miles away (3*275*2 = 1650 miles at $0.575/mi = $545 and require 6 per-diem @$28/day = $168 and 6 hotel nights at $60/night=$360) for a Central-Eastern total of $1073/year; and the Northern sites are ~300 miles away (3*300*2 = 1800 at $0.575/mi = $1035, and require 6 per-diem @$28/day = $168 and 6 hotel nights @$60/night = $360) for a Northern total of $1,563/year. The grand total for field work travel will be $2,892.50/year, i.e. $5,785 for two years. The remaining funds will be used for separate trips to the two herpetological fairs and to pet stores in the Chicago area (Amtrak train tickets, per-diem). We will use the current SIUC Office of Sponsored Projects Administration reimbursement rate for mileage found at http://www.siu.edu/orda/rates/. We will use the Volunteer Time Sheet & Mileage Record to keep track of mileage. The Office of the Vice Chancellor for Research at SIUC will contribute the remaining $1991 for travel expenses as match.

Equipment: No equipment purchase is necessary for this project. The following equipment is available in the Catenazzi Lab: real time PCR machine (Step One Plus), centrifuges, dry bath, laminar hood, incubators, -20C and -80C freezers.

Materials and supplies: We request $3,002 in funds to purchase commodities, swabs, standards and reagents required to perform PCR analyses and to conduct infection experiments. The Department of Zoology at SIUC will contribute the remaining $5,881 for materials and supplies as match.

Quantitative PCR analyses will be done in the Catenazzi Lab at SIUC. The PI will train undergraduate student workers and volunteers in the DNA extraction and real-time PCR protocol. On the basis of previous experience, the average cost per sample including swab, vials, extraction and PCR reagents (excluding labor) is ~$6.5. Thus, we estimate to be able to process at least 1250 swab samples (~$8,200). This is the list of supplies that will be purchased for PCR analyses: 8 kits of Prepman Ultra #4318930, 1 kit @126 (total $1,008), 1 kit @$126; 1 set of Taqman MasterMix #4364340, 5 x 5ml @$2,160 (total $1,512); 1 set of Taqman MasterMix #4364338, 2 x 5ml @$2,160 (total $916); 3 TaqMan MGB Probe (1 for Bd, 1 for Bsal, 1 for Rv) #4316032, 1 @$693 (total $2,079), 15 boxes of 96-well Fast Thermal Cycling Plates #4346907, 1 box @$37.89 (total $568.35); 1 box of fine-tip sterile swabs #MW-113, 1250 @$300 (total $300); 5 boxes of FisherBrand vials #02-707-352, 500 @$140.02 (total $420.06); 2 cases of 2-20 ul pipette tips #02-707-432, 1 case @$242.15 (total $484.30); the grand total for PCR analyses is $7,287.71. This amount is approximate and may vary depending on price increases and discounts.

The remaining $1,595 will be used to purchase commodities and supplies to rear *Ambystoma* salamanders from field-collected eggs and to conduct the infection trials (PCR analyses needed to assess infection status during the trials are included in the $7,287.71 explained above). These commodities include culturing supplies to grow Bsal (Petri dishes, flasks, agar, tryptone, glucose, etc), aquaria and terraria for developing larvae and young, live food, and jars.

Contractual Services: N/A

Multipurpose projects: N/A

Relationship with other grants: The salamander-killing fungus was only described two years ago, and no previous study has documented the distribution of this pathogen in Illinois or in the United States. Very few laboratories in the USA and Europe currently have the ability to perform real time PCR analyses to quantify infection with *Bsal*. Therefore, our study will be the first in Illinois and among the first in the United States to investigates the presence and distribution of *Bsal* at field sites and among salamander pets, and to assess the susceptibility of native species to this virulent fungal disease.

A previously funded grant (Project number T-56-R1, PI Karen Lips) surveyed the historical and current distribution of *Batrachochytrium dendrobatidis* (*Bd*) in Illinois. The study surveyed 1028 anuran museum specimens for *Bd*, and found that the oldest infected specimen was a *Lithobates sphenocephala* collected in 1888. Moreover, *Bd* was geographically widespread throughout Illinois by 1900, and a *Bd* prevalence shift in the 1940s may indicate a historic epizootic event. The study also found that *Bd* remains widespread in Illinois, with prevalence varying across species and seasons, but no indication that the fungus is causing significant population declines.

Timeline: Significant milestones for the project are:
December 2015 – March 2016: field surveys and skin swabs of salamanders.
July 2016: annual report completed
November 2017 – March 2017: field surveys and skin swabs of salamanders, collection of eggs.
July 2017: annual report completed
July 2017 – August 2017: infection experiments
January 2018: PCR analyses completed
June 2018: final report completed

General:
(i) Substantial in Character and Design
The project statement describes a need consistent with the State Wildlife Grants (SWG), specifically items in the Illinois Wildlife Conservation Plan that cover Wetlands Campaign, Invasive Species, Monitoring and Research of Wildlife Disease, and Monitoring and Research on Amphibian Species of Conservation Priority (http://dnr.state.il.us/ORC/WildlifeResources/theplan/final/).]; states a purpose and sets objectives, both of which are based on the need; uses a planned approach, appropriate procedures; research; and is cost effective.

(ii) Compliance:
This project will require the following permits: scientific collecting permit, endangered species permit, permit to access state site, permit to access nature preserves, permit to access forest service area. All permits will be requested and no work will be conducted before obtaining all necessary authorizations at each site.

The IDNR will use its CERP (Comprehensive Environmental Review Process) as a tool to aid the Department in meeting NEPA compliance for the projects outlined under this grant proposal. It is the Department's policy to require CERP applications for all land disturbing activities unless those activities are covered by CERP exemptions (see the enclosed Comprehensive Environmental Review Process documents).

This project requests a Categorical Exclusion 1.4B1 because its purpose is research, inventory and information collection activities which involve negligible animal mortality or habitat destruction, no introduction of contaminants, and no introduction of non-indigenous organisms.

All planned activities will also be in compliance with the Endangered Species Act. All determinations and documentation will in accordance with the current established U. S. Fish and Wildlife Service protocols for Section 7.

All planned activities will be in compliance with the National Historic Preservation Act and the Council on Historic Preservation Act. All determinations and documentation will be in accordance with the terms of the Programmatic Agreement, as amended, effective September 23, 2002.

When applicable, those planned activities which involve a floodplain and/or jurisdiction wetlands will be done in accordance with Presidential Executive Orders 11988 and 11990.

References

Blooi, M., A. Martel, F. Haesebrouck, F. Vercammen, D. Bonte, and F. Pasmans. 2015. Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. Scientific Reports **5**:8037.

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Kilpatrick, A. M., C. J. Briggs, and P. Daszak. 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends in Ecology & Evolution **25**:109-118.

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Martel, A., M. Blooi, C. Adriaensen, P. Van Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, B. R. Schmidt, U. Tobler, K. Goka, K. R. Lips, C. Muletz, K. R. Zamudio, J. Bosch, S. Loetters, E. Wombwell, T. W. J. Garner, A. A. Cunningham, A. Spitzen-van der Sluijs, S. Salvidio, R. Ducatelle, K. Nishikawa, T. T. Nguyen, J. E. Kolby, I. Van Bocxlaer, F. Bossuyt, and F. Pasmans. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science **346**:630-631.

Martel, A., A. Spitzen-van der Sluijs, M. Blooi, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, K. Chiers, F. Bossuyt, and F. Pasmans. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences of the United States of America **110**:15325-15329.

Spitzen-van der Sluijs, A., F. Spikmans, W. Bosman, M. de Zeeuw, T. van der Meij, E. Goverse, M. Kik, F. Pasmans, and A. Martel. 2013. Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. Amphibia-Reptilia **34**:233-239.

Documents supporting the grant Proposal:

The following are attached in support of this grant proposal:
Application for federal assistance (form 424)
Comprehensive Environmental Review Process
Federal aid section 7 evaluation form
Grant proposal budget
Illinois Clearinghouse response per federal executive order 12372
Figures
Appendix 1: Species List

Volunteer Time Sheet & Mileage Record
NEPA compliance checklist

Figures

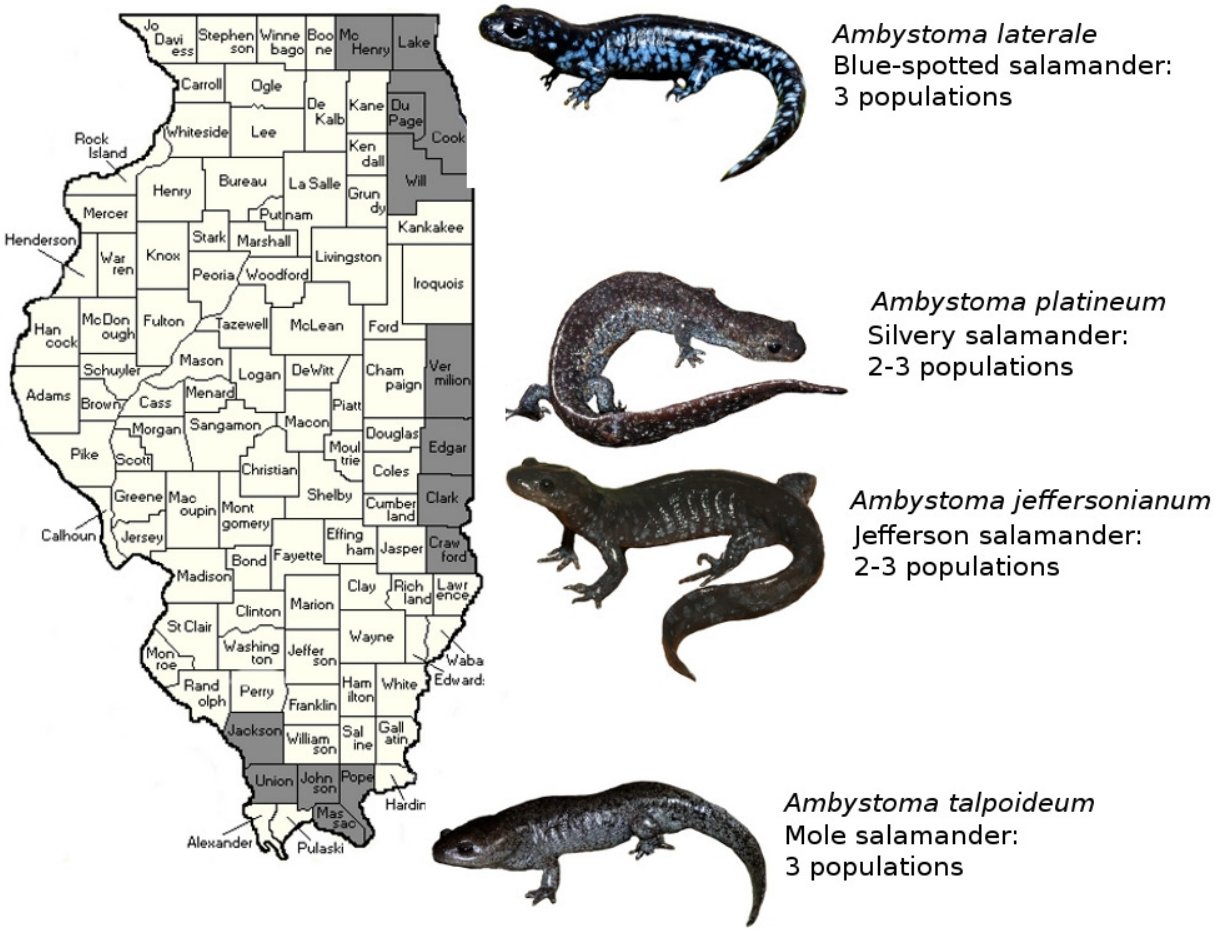

Figure 1. Map of the State of Illinois with the three study regions (highlighted counties) hosting populations of the four species of *Ambystoma* of greatest concern in Illinois. Photo credits: *A. laterale*: John P. Clare; *A. platineum*: Matt Ignoffo; *A. jeffersonianum*: Todd Pierson; *A. talpoideum*: Matthew Niemiller.

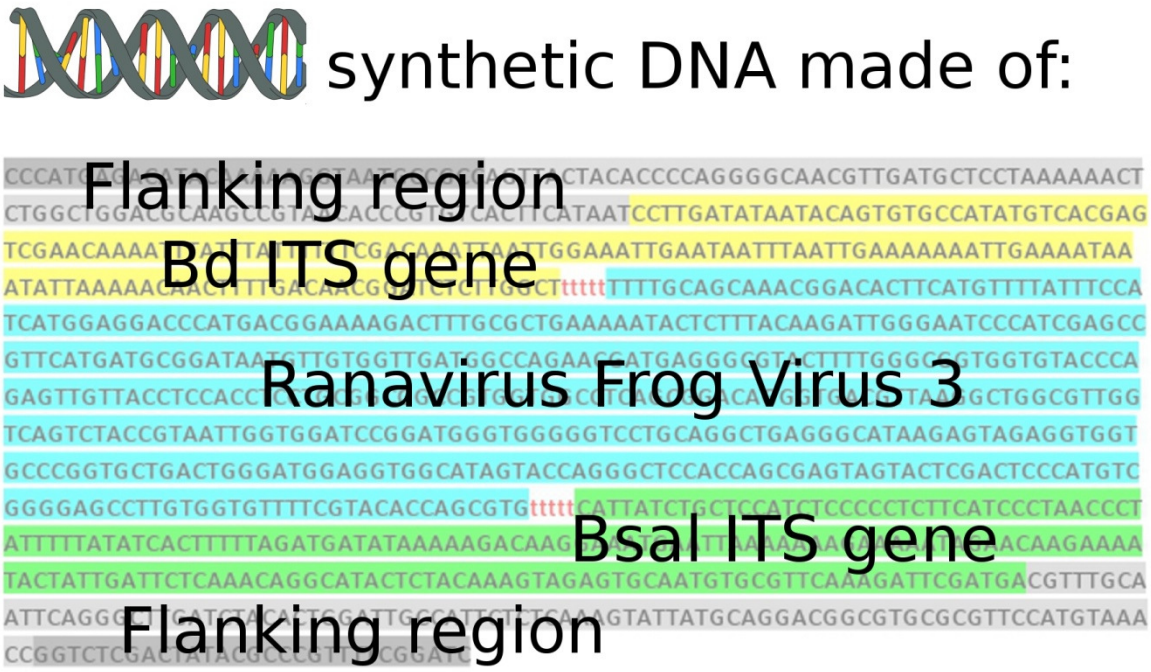

Figure 2. Cost-effective protocol uses a sequence of synthetic, double-stranded DNA that allows simultaneous quantification of infection by *Batrachochytrium salamandrivorans* (*Bsal*), *B. dendrobatidis* (*Bd*) and *Ranavirus* in a single PCR reaction. The sequence is used as standard during real time PCR. The following gene sequences are used, for *Bsal*: ITS-1 & 5.8S (Blooi et al. 2013, Genbank KC762295);for *Bd*: ITS-1 & 5.8S (Boyle et al. 2004, Genbank AY598034); for Ranavirus Frog Virus 3 (Mao et al. 1996, GenBank: DQ906049.1).

Appendix 1

Species of salamanders in Illinois (*Species of Greatest Concern)

Ambystomatidae
**Ambystoma jeffersonianum*, Jefferson Salamander
**Ambystoma laterale*, Blue-spotted Salamander
Ambystoma maculatum, Spotted Salamander
Ambystoma opacum, Marbled Salamander
**Ambystoma platineum*, Silvery Salamander
**Ambystoma talpoideum*, Mole Salamander
Ambystoma texanum, Small-Mouthed Salamander
Ambystoma tigrinum, Eastern Tiger Salamander

Cryptobranchidae
*Cryptobranchus alleganiensis, Hellbender

Plethodontidae
**Desmognathus conanti*, Spotted Dusky Salamander
Eurycea cirrigera, Southern Two-lined Salamander
Eurycea longicauda, Long-tailed Salamander
Eurycea lucifuga, Cave Salamander
**Hemidactylium scutatum*, Four-toed Salamander
Plethodon cinereus, Eastern Red-backed Salamander
Plethodon dorsalis, Northern Zigzag Salamander
Plethodon glutinosus, Northern Slimy Salamander

Proteidae
**Necturus maculosus*, Mudpuppy

Salamandridae
Notophthalmus viridescens, Eastern Newt

Sirenidae
Siren intermedia, Lesser siren